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Abstract: Periodontitis is a bacterially-induced oral inflammatory disease that is characterised by tissue degradation and bone loss. Porphyromonas gingivalis is a gram negative bacterial species highly associated with the pathogenesis of chronic periodontitis. Receptor activator of nuclear factor-kB ligand (RANKL) induces bone resorption whilst osteoprotegerin (OPG) is a decoy receptor that blocks this process. Cyclooxygenase-2 (COX-2) is an enzyme responsible for the production of prostaglandin (PGE)(2,) which is a major inflammatory mediator of bone resorption. Mitogen-activated protein kinases (MAPK) are intracellular signalling molecules involved in various cell processes, including inflammation. This study aimed to investigate the effect of P. gingivalis on MAPKs and their involvement in the regulation of RANKL, OPG and COX-2 expression in bone marrow stromal cells. P. gingivalis challenge resulted in the phosphorylation of primarily the p38 MAPK. RANKL and COX-2 mRNA expressions were up-regulated, whereas OPG was down-regulated by P. gingivalis. The p38 synthetic inhibitor SB203580 abolished the P. gingivalis-induced RANKL and COX-2 expression, but did not affect OPG. Collectively, these results suggest that the p38 MAPK pathway is involved in the induction of RANKL and COX-2 by P. gingivalis, providing further insights into the pathogenic mechanisms of periodontitis.

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Porphyromonas gingivalis induces RANKL in bone marrow stromal cells: involvement of the p38 MAPK

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Abstract

Periodontitis is a bacterially-induced oral inflammatory disease that is characterised by tissue degradation and bone loss. *Porphyromonas gingivalis* is a gram negative bacterial species highly associated with the pathogenesis of chronic periodontitis. Receptor activator of nuclear factor-kB ligand (RANKL) induces bone resorption whilst osteoprotegerin (OPG) is a decoy receptor that blocks this process. Cyclooxygenase-2 (COX-2) is an enzyme responsible for the production of prostaglandin $(PGE)_{2,}$ which is a major inflammatory mediator of bone resorption. Mitogen-activated protein kinases (MAPK) are intracellular signalling molecules involved in various cell processes, including inflammation. This study aimed to investigate the effect of *P. gingivalis* on MAPKs and their involvement in the regulation of RANKL, OPG and COX-2 expression in bone marrow stromal cells. *P. gingivalis* challenge resulted in the phosphorylation of primarily the p38 MAPK. RANKL and COX-2 mRNA expressions were up-regulated, whereas OPG was down-regulated by *P. gingivalis*. The p38 synthetic inhibitor SB203580 abolished the *P. gingivalis*-induced RANKL and COX-2 expression, but did not affect OPG. Collectively, these results suggest that the p38 MAPK pathway is involved in the induction of RANKL and COX-2 by *P. gingivalis*, providing further insights into the pathogenic mechanisms of periodontitis.

Keywords

Porphyromonas gingivalis, Bone marrow stromal cells, Receptor activator of nuclear factor-kB ligand, Osteoprotegerin, Cyclooxygenase -2, p38 MAPK.

1. Introduction

Periodontitis is a bacterially-induced inflammatory disease that destroys the tooth-supporting (periodontal) tissues. Due to the resulting inflammation, the periodontal connective tissue is degraded and underlying alveolar bone destroyed, eventually leading to tooth loss if the disease is left untreated. *Porphyromonas gingivalis* is a Gram-negative black-pigmenting anaerobe that is strongly associated with chronic periodontitis [1]. It is postulated that *P. gingivalis* can contribute to tissue and bone destruction in periodontitis, by its capacity to express a number of virulence factors including lipopolysaccharide (LPS) and gingipains [2, 3].

Under physiological conditions, the tight balance between bone resorption and bone formation in the adult skeleton determines the levels of bone mass. When the coupling of these two processes is disturbed in favor of enhanced bone resorption, this can lead to net bone loss in pathological conditions, including osteoporosis, rheumatoid arthritis and periodontitis [4]. Bone resorption is the process of degradation of bone by specialised cells called osteoclasts. The control of this process is dependent on the molecular interplay of the receptor activator of nuclear factor-kB ligand (RANKL) and osteoprotegerin (OPG). RANKL is a member of the tumor necrosis factor (TNF) ligand family and was identified as the type II membrane-bound protein ligand that triggers osteoclast differentiation and activation through its cognate RANK receptor on osteoclast precursors [5-8]. RANKL is expressed by osteoblasts, stromal cells [9], and activated T- and B-cells [10]. Hormones, such as parathyroid hormone (PTH), 1,25-dihydroxy vitamin D3, and prostaglandin E_2 (PGE_2) [11], and cytokines such as TNF-α, Interleukin(IL)-1β and IL-6 all have the potential to induce the expression of RANKL [12]. *P. gingivalis* is

known to induce RANKL expression in osteoblasts [13], gingival fibroblasts and periodontal ligament cells [14], bone marrow stromal cells [15] and T-cells [16]. OPG is a secreted protein that acts as a decoy receptor for RANKL. Thus, it inhibits bone resorption by binding to RANKL and preventing its interaction with its cognate receptor RANK [17, 18]. OPG is constitutively expressed at high levels by osteoblasts, stromal cells, endothelial cells and fibroblasts [19].

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine specific protein kinases, including p38, c-Jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK) and extracellular signal-related kinases (ERK1/2; also known as p44/p42 MAPK). They are important molecules in signalling cascades involved in cell differentiation, proliferation and cell death [20-22], and are activated through phosphorylation of tyrosine/threonine residues in signal transduction cascades. Once a MAPK is activated, transcription factors, RNA-binding proteins and other kinases may be phosphorylated, initiating events such as gene expression and post-translational protein modifications.

There is evidence that MAPKs are implicated in RANKL, OPG and cyclooxygenase (COX)-2 regulation [23-27], and that MAPK signalling cascades can be activated in response to *P. gingivalis* infection [13, 28]. Although previous works investigated the regulation of MAPKs in response to *P. gingivalis*, or the effects of *P. gingivalis* on RANKL, OPG and COX-2 expression, the putative cross-talks of these regulatory pathways are not fully understood. This study aims to elucidate the involvement of MAPK signalling pathways in the regulation of RANKL, OPG and COX-2 expression in bone marrow stromal cells, by *P. gingivalis*.

2. Materials and methods

2.1 Bacterial growth conditions and characterisation of bacterial supernatant

Porphyromonas gingivalis W50 strain was grown on blood agar plates supplemented with 5% horse blood in an anaerobic environment containing 80% nitrogen, 10% hydrogen and 10% CO_2 at 37°C. This was then sub-cultured into 10 ml of media consisting of brain heart infusion (BHI) broth supplemented with 5 µg/ml of hemin. The following day, the 10 ml culture was inoculated into 90 ml of fresh media and allowed to grow for 6 days. At day 6, the liquid cultures were centrifuged at 8500 rpm for 45 min at 4ºC. The resulting culture supernatants were collected, aliquoted and stored at -80°C, to be used for further experimentation. Total bacterial protein concentration in these *P. gingivalis* culture supernatants was determined using the Bio-Rad Protein Assay, whereas the endotoxin as well as Arg-X and Lys-X protease activities were determined as previously described [15]

2.2 Cell culture

The murine osteogenic cell-line W20-17 was used for this study. Cells were cultured in alpha minimum essential medium, supplemented with 10% v/v foetal bovine serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco-BRL), at 37º C and 5% CO_2. Cells were seeded at a density of 2 x 10^4 cells/cm^2 and allowed to attach overnight. The cell cultures were then challenged with *P. gingivalis* W50 culture supernatants (5 µg/ml), for up to 24 h. Each group was performed in duplicate cultures, which were then pooled together. For investigations involving the inhibition of p38, cells were pre-

incubated for 1 h with 10 μM of the chemical inhibitor of p38 SB203580 (Promega), prior to challenged with *P. gingivalis*.

2.3 Extraction of total RNA and synthesis of cDNA

Total RNA was extracted from cells using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The RNA was then quantified using a Nanodrop spectrophotrometer and reversed transcribed into cDNA. For this purpose, 1 μg of total RNA was incubated with 0.5 μg/ml of oligo dT primer (Promega) at 70°C for 5 min and cooled on ice. A master mix was then added to samples, comprising of 10mM dNTPs, 200 units of moloney murine leukemia virus (M-MLV) reverse-transcriptase enzyme and buffer (Promega), and dH_20 to a final volume of 25 μl. For the reverse-transcription reaction, these samples were incubated at 40°C for 60 min, 70°C for 15 min and cooled down to 4°C.

2.4 Quantitative real-time PCR (qPCR)

To quantify the mRNA expression levels, qPCR was performed on the prepared cDNA samples. TaqMan® Gene Expression Assays (Applied Biosystems) and ROX qPCR mastermix were used for the amplification reactions. The qPCR analyses were perfomed in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). To normalise Ct values, eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2) and cytochrome 1 (CYC1) housekeeping genes were used. The Applied Biosystems assay IDs for the target genes are TNFSF11 (RANKL): Mm00441908_m1, TNFRSF11B (OPG): Mm00435452_m1, COX-2: Mm00478377_g1, EIF4A2: Mm00834357_g1 and CYC1:

Mm00470540_g1. The amplification conditions were 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min.

2.5 Extraction of intracellular protein from cell lysates

After indicated time-points of challenge with *P. gingivalis* supernatants, the cell monolayers were washed with cold 1 x PBS and then lysed using chilled radio-immunoprecipitation assay (RIPA) buffer, consisting of 150 mM sodium dodecyl sulphate (SDS), 50mM Tris pH 7.3, 2.5% sodium chloride, 1% NP-40 and 1:100 Protease Inhibitor Cocktail (Sigma). Just prior to addition to the cell monolayers, 10^{-1} M sodium orthovanadate (Sigma) and 10^{-2} x 5 M sodium fluoride (Sigma) were added to the buffer. To obtain the cell lysates, the cell monolayers were incubated in RIPA buffer for 20 min at 4 ºC. Lysates were then collected and centrifuged at 1500 rpm for 20 min at 4 ºC, and the pelleted cell debris was discarded. To achieve complete homogenisation, these cell lysates were passaged through a 21G needle. Total protein concentration was then determined using the Pierce BCA Kit, according to the manufacturers instructions.

2.6 Western immunoblotting

Western immunoblotting was used to evaluate the total and phosphorylated levels of the three studied MAPK, namely p38, p44/42, and SAPK/JNK. For this purpose, 6 µg of total protein from each lysate was used. The samples were denatured for 5 min at 100°C and separated on a 4% - 12% Bis-Tris-HCl polyacrylamide gradient gel (Invitrogen), at 100 V for 2 h. The separated protein content was electrotransferred from the gel onto a polyvinylidene fluoride (PVDF) membrane, at 100 V for 1 h. The membranes were then

blocked (1 x Tris-buffered-saline, including 5% milk and 0.05% Tween) for 1 h at room temperature, and then incubated overnight at 4°C with appropriate primary antibodies, purchased from Cell Signalling Technologies (phospho-p44/42 rabbit antbody - Cat. No. 4370, phospho-p38 rabbit antibody - Cat. No. 9211, phospho-SAPK/JNK rabbit antibody - Cat. No. 4668, total p44/42 rabbit antibody - Cat No. 4695, total p38 antibody - Cat. No. 9212, total SAPK/JNK antibody - Cat. No. 9258). These primary antibodies were detected by using an anti-rabbit IgG horse radish peroxide-conjugated secondary antibody (Cat. No. 7074, Cell Signalling Technology). The protein bands were finally visualised by short incubation of the membrane in ECL chemiluminescent substrate (GE Healthcare), followed by exposure of the membrane onto a photographic film, where the signal was developed.

2.7 Statistical analyses

The gene expression data was analysed using a one-way analysis of variance (ANOVA), using Bonferroni post-hoc test to further compare differences between the groups. All statistical analyses were performed on GraphPad Prism.

3. Results

3.1 Effects of *P. gingivalis* on MAPK signalling proteins p44/42, SAPK/JNK and p38.

To investigate the effect of *P. gingivalis* on the three MAPKs, the cells were cultured in the presence or absence of 5µg/ml *P. gingivalis* W50 culture supernatants for 60 min. Both total and phosphorylated levels of MAPK were investigated by Western

immunoblotting after 0, 15, 30 and 60 min of challenge with *P. gingivalis*. The phosphorylation of all three SAPK/JNK, p44/42, and p38 proteins was up-regulated after 15 min in both *P. gingivalis*-challenged and unchallenged cell cultures, while the total level of these proteins remained unchanged (Fig. 1). Of note, the MAPK phosphorylation observed in unchallenged cells is a likely result of stimulation by FBS, due to the replenishment of the cell culture media. As FBS-free media resulted in very low RANKL gene expression by the cells (data not shown), this protocol was not considered for further experimentation.

Differences in phosphorylation of these proteins between the *P. gingivalis*-challenged and unchallenged groups were further considered. In the case of p44/p42, there was no obvious change in phosphorylation levels between the *P. gingivalis*-challenged and the control groups, at any time-point (Fig. 1A). When phosphorylation of JNK was investigated, no differences were evident up to 30 min, but there appeared to be a slight up-regulation in cells challenged with *P. gingivalis,* with a 1.5 fold increase after 60 min, compared to the corresponding 60 min unchallenged control, as measured by densitometry (Fig. 1B). In contrast, a strong phosphorylation of p38 was evident at 30 min in *P. gingivalis*-challenged cells compared to the corresponding 30 min control, which was further increased by a 2.1 fold increase at 60 min (Fig. 1C). Hence, within the limitations of densitometric measurements due to potential saturation of the bands, these findings indicate that p38 is the MAPK most affected by *P. gingivalis*.

3.2 Effects of p38 inhibition on RANKL, OPG and COX-2 gene expression

To investigate the involvement of p38 in the regulation of RANKL, OPG and COX-2 the chemical inhibitor of p38 (SB203580) was used. W20-17 bone marrow stromal cells were pre-incubated with the inhibitor (10μM) and then challenged with *P. gingivalis* (5μg/ml) for 24 h. RANKL gene expression was significantly up-regulated in *P. gingivalis*–challenged cells compared to the unchallenged control (Fig. 2), in line with previous observations [15]. When the cells were pre-treated with the p38 inhibitor, a significant decrease was observed in the capacity of *P. gingivalis* to induce RANKL expression (Fig. 2), accounting for an 83% reduction compared to non-pre-treated cells.

With regard to OPG gene expression, this was down-regulated in response to *P. gingivalis*, after 24 h (Fig. 3). Pre-treatment with the p38 inhibitor did not rescue the down-regulation of OPG expression caused by *P. gingivalis*, and in fact, this caused a further down-regulation, which did not prove to be significant (Fig. 3). In the absence of *P. gingivalis*, pre-treatment of the cells with the p38 inhibitor alone caused a down-regulation in OPG expression relative to the control but this was not significant either.

Finally, the gene expression of COX-2, an enzyme responsible for PGE_2 production, was also investigated. *P. gingivalis* culture supernatants significantly enhanced COX-2 expression after 24 h of challenge (Fig. 4). Interestingly, the p38 inhibitor significantly reduced the *P. gingivalis*-induced COX-2 expression in the cells by 76 %, in a trend similar to that of RANKL inhibition (Fig. 4).

4. Discussion

To understand the pathogenesis of periodontitis, it is important to identify the functional role of signalling pathways that are activated by *P. gingivalis*, such as Activator Protein (AP)-1, MAPKs and NF-κB in various cell types [13, 28-30]. In this study, we used a previously established model in bone marrow stromal cells [15], to investigate the putative involvement of MAPK signalling pathways, including p38, JNK and p44/42, in the regulation of RANKL, OPG and COX-2 expression, in response to *P. gingivalis* challenge. Phosphorylation of p38 and JNK were notably increased after 60 min of challenge with *P. gingivalis*, concurring with previous results [28, 30]. In contrast, the phosphorylation of p44/42 did not appear to be affected and was not explored further. The increase in p38 phosphorylation was considerably stronger than that observed for JNK. Although the involvement of JNK may not be necessarily be excluded, under the present indications the p38 MAPK pathway was further investigated in relation to the regulation of RANKL, OPG and COX-2 expression. Previous studies have shown that this pathway can directly or indirectly regulate inflammatory regulators, such as IL-1β, TNF-α, RANKL and PGE_2 [25, 27, 31].

The involvement of the p38 MAPK pathway in RANKL, OPG and COX-2 gene expressions was investigated by the use of the specific pharmacological inhibitor SB203580. This treatment significantly abrogated *P. gingivalis*-induced RANKL expression, although OPG regulation was unaffected. In line with the present findings, it has been shown that *Streptococcus pyogenes* can induce RANKL through the p38 MAPK pathway in osteoblasts [26]. In contrast, AP-1 is suggested to mediate RANKL induction in the same cells, in response to infection with viable *P. gingivalis* [13]. This variation

could be attributed to the cell type studied, the *P. gingivalis* strain used, or the potential differential responses to viable bacteria versus secreted bacterial products.

The enzyme COX-2 is responsible for the conversion of arachidonic acid to PGE_2. We have previously established in this *in vitro* experimental model that RANKL up-regulation is partly mediated by PGE_2, following COX-2 expression [15]. It was recently shown that IL-1β induces RANKL in human periodontal ligament cells, mediated by PGE_2 production, possibly through all three MAPKs [23], although others implicate mainly the ERK (p44/42) MAPK in this PGE_2-mediated event [24]. In the present study in bone marrow stromal cells, *P. gingivalis*-induced COX-2 expression was significantly down-regulated with the p38 inhibitor, indicating that the p38 MAPK is implicated in this induction.

In this study, *P. gingivalis* culture supernatant was employed, which may better reflect the *in vivo* situation. The deeply located bone marrow stromal cells are more likely to be encounter by various *P. gingivalis* components, rather than whole viable cells. Other works have concentrated on using purified *P. gingivalis* LPS to investigate signalling pathways that may be activated. For instance, it has been shown that challenge with *P. gingivalis* LPS causes an increase in phosphorylated p38 in human monocytes, but not endothelial cells [32]. In addition, in another experimental system, challenge with purified *Aggregatibacter actinomycetemcomitans* and *Escherichia coli* LPS also up-regulated RANKL expression through the p38 MAPK pathway, in line with the present findings [33]. However, another study demonstrated that these two LPS induced RANKL expression in murine osteoblasts, mediated by the ERK MAPK pathway [34]. These discrepancies highlight the utilisation of different signalling pathways by different cell

types, in response to similar challenges. They also underline that the virulence factors of bacteria in the oral biofilm could target a multitude of cell types and signalling pathways in the host. It is possible in the present study that *P. gingivalis* LPS may be activating the p38 MAPK pathway, and subsequently regulating of COX-2 and RANKL expression. To this extent, in our previous work, removal of LPS prevented *P. gingivalis* culture supernatant from inducing RANKL and COX-2 expression, indicating that LPS is a key virulence factor in these events associated with the induction of bone resorption [15]. Although isolated virulence factors have not been used in this study, it may be a more representative approach to use bacterial culture supernatant to mimic the disease microenvironment *in vitro*, as this could take into account potential synergistic effects among virulence factors.

The involvement of the p38 MAPK pathway in periodontitis could allow the use of specific inhibitors for therapeutic purposes, as demonstrated in *in vivo* experimentations. One study showed that in LPS-induced alveolar bone resorption, p38 inhibition reduced bone destruction in an *in vivo* periodontitis model [35, 36]. Through inhibiting p38α, bone resorption was also reduced in a rheumatoid arthritis model in rats [31]. Such studies all support a potential use of p38 inhibitors in the treatment of chronic periodontitis.

5. Conclusion

This data suggests that *P. gingivalis* induces COX-2 and RANKL expression in bone marrow stromal cells, and that the p38 MAPK pathway is involved in this process. This

provides further insights to the pathogenesis of *P. gingivalis*-associated periodontitis, and supports the inhibition of p38 for therapeutic purposes in the disease.

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Figure legends

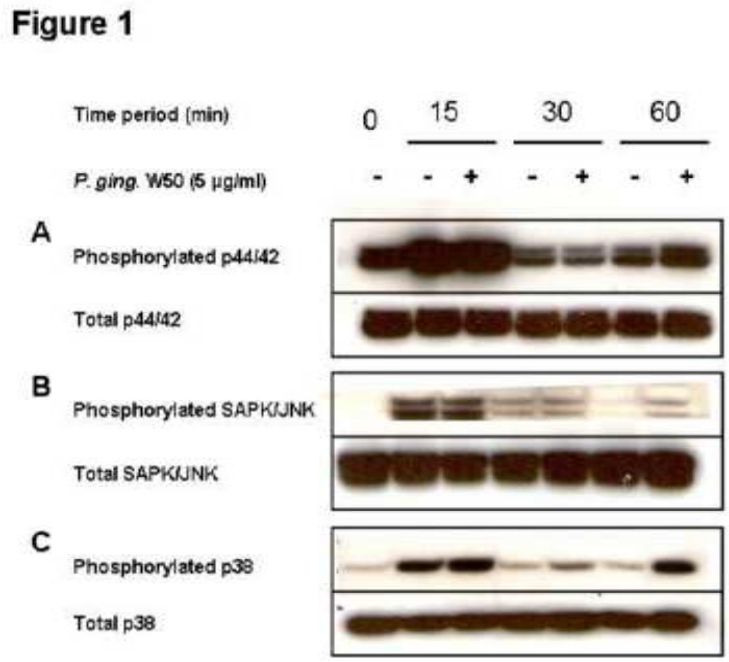

Figure 1. Western immunoblotting of phosphorylated and total p44/42, SAPK/JNK and p38. In these experiments, W20-17 bone marrow stromal cells were cultured in the presence or absence of 5 µg/ml protein concentration of *P. gingivalis* W50 culture supernatants. Cell lysates were obtained at 0, 15, 30 and 60 min from the *P. gingivalis*-challenged cultures and the corresponding unchallenged controls. Western immunoblotting was then performed to determine the total and phosphorylated levels of p44/42 (A), SAPK/JNK (B), and p38 (C).

Figure 2

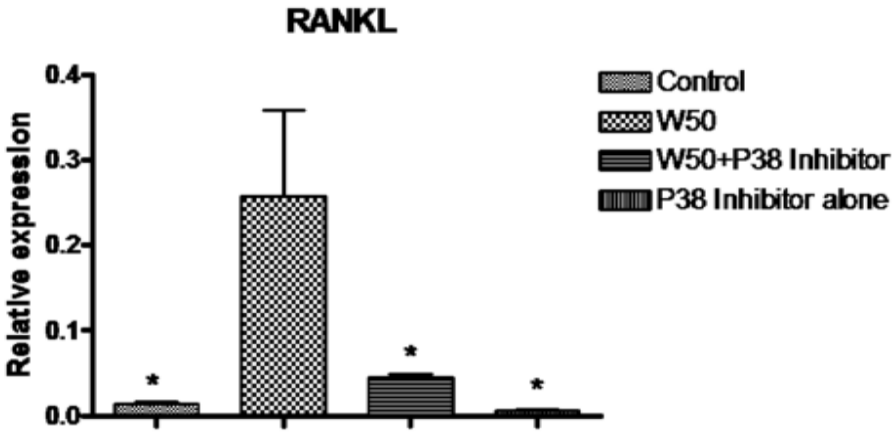

Figure 2. Effect of p38 inhibitor on RANKL expression in response to *P. gingivalis*. W20-17 cells were either pre-treated or non-treated with the p38 inhibitor SB203580 (10^{-5} M) for 1 h. The cells were then challenged with 5 µg/ml protein concentration of *P. gingivalis* W50 culture supernatants for 24 h. The gene expression levels of RANKL were measured by qPCR analysis, and the results are expressed as the $2^{-\Delta CT}$ formula, normalised against the average housekeeping gene expression in each sample. Bars represent mean values ± SEM from three independent experiments. A one way (ANOVA) and Bonferroni post-hoc test were used to compare differences between the *P. gingivalis*-challenged group and the other groups (*$p < 0.05$).

Figure 3

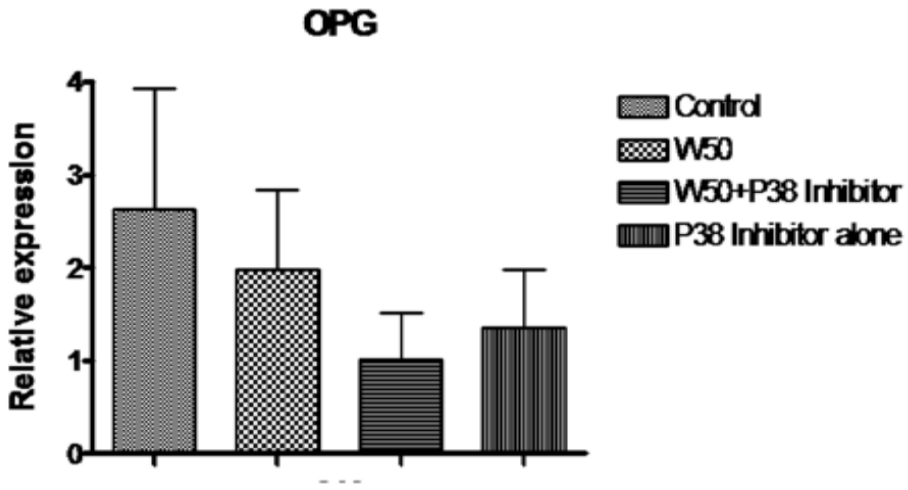

Figure 3. Effect of p38 inhibitor on OPG expression in response to *P. gingivalis*. W20-17 cells were either pre-treated or non-treated with the p38 inhibitor SB203580 (10^{-5} M) for 1 h. The cells were then challenged with 5 µg/ml protein concentration of *P. gingivalis* W50 culture supernatants for 24 h. The gene expression levels of OPG were measured by qPCR analysis, and the results are expressed as the $2^{-\Delta CT}$ formula, normalised against the average housekeeping gene expression in each sample. Bars represent mean values ± SEM from three independent experiments. A one way (ANOVA) and Bonferroni post-hoc test were used to compare differences between the *P. gingivalis*-challenged group and the other groups (*$p < 0.05$).

Figure 4

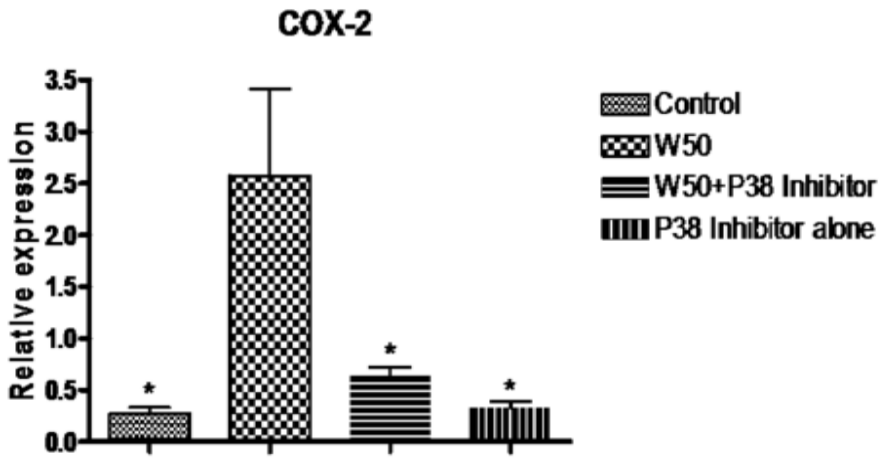

Figure 4. Effect of p38 inhibitor on COX-2 expression in response to *P. gingivalis*. W20-17 cells were either pre-treated or non-treated with the p38 inhibitor SB203580 (10^{-5} M) for 1 h. The cells were then challenged with 5 µg/ml protein concentration of *P. gingivalis* W50 culture supernatants for 24 h. The gene expression levels of OPG were measured by qPCR analysis, and the results are expressed as the $2^{-\Delta CT}$ formula, normalised against the average housekeeping gene expression in each sample. Bars represent mean values ± SEM from three independent experiments. A one way (ANOVA) and Bonferroni post-hoc test were used to compare differences between the *P. gingivalis*-challenged group and the other groups (*$p < 0.05$).